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Formation of Precise Connections in the Olfactory Bulb Occurs in the Absence of Odorant-Evoked Neuronal Activity

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Summary

Olfactory neurons expressing the same odorant receptor converge to a small number of glomeruli in the olfactory bulb. In turn, mitral and tufted cells receive and relay this information to higher cortical regions. In other sensory systems, correlated neuronal activity is thought to refine synaptic connections during development. We asked whether the pattern of connections between olfactory sensory axons and mitral cell dendrites is affected when odor-evoked signaling is eliminated in mice lacking functional olfactory cyclic nucleotide–gated (CNG) channels. We demonstrate that olfactory sensory axons converge normally in the CNG channel mutant background. We further show that the pruning of mitral cell dendrites, although slowed during development, is ultimately unperturbed in mutant animals. Thus, the olfactory CNG channel—and by inference correlated neural activity—is not required for generating synaptic specificity in the olfactory bulb.

Introduction

In sensory systems, primary neurons in the periphery project axons to precise locations within the brain to generate a sensory map. This internal representation of the external world then translates stimulus features into a neural code that allows the discrimination of complex sensory information. It has been suggested that the development of sensory maps requires guidance molecules to generate a coarse pattern of innervation that is subsequently refined by activity-dependent processes (reviewed by Shatz, 1990; Goodman and Shatz, 1993; Katz and Shatz, 1996; Tessier-Lavigne and Goodman, 1996). In the visual system, for example, complementary gradients of ephrin receptors on retinal afferents and ephrins expressed by the target cells in the brain are thought to provide positional cues dictating the formation of a precise retinotopic map (Feldheim et al., 1998; Frisén et al., 1998). These data provide compelling evidence for the role of molecular cues in establishing a precise pattern of synaptic connections. However, the role of neuronal activity in this process has been controversial (e.g., see Crair et al., 1998; Crowley and Katz, 1999; Hubener and Bonhoeffer, 1999; Weliky and Katz, 1999).

What is the role of activity-dependent processes in the establishment of precise synaptic connections in the olfactory system? Odorant stimuli are detected by receptors expressed by olfactory neurons in the olfactory epithelium (reviewed by Shepherd, 1994; Buck, 1996). Each sensory neuron projects a single unbranched axon to the brain, where it synapses with the dendrites of projection neurons and interneurons of the olfactory bulb. In the mammalian bulb, these synapses are confined within ~1800 discrete and circumscribed loci, the glomeruli. Individual olfactory neurons are thought to express only one of the ~1000 odorant receptor genes encoded in the genome (Ngai et al., 1993; Buck, 1996; Barth et al., 1997; Malnic et al., 1999). Neurons expressing a given receptor are dispersed broadly over the sensory epithelium yet converge upon a small number of spatially invariant glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998). This pattern of innervation in the olfactory bulb is thought to provide the anatomical basis for an olfactory sensory map.

Within each glomerulus, olfactory neuron axons form synapses with the dendrites of mitral cells, the primary projection neurons in the bulb (Mori, 1987). In the adult mouse, most mitral cells extend a single primary radial dendrite into the glomerular layer that ramifies extensively within a single glomerulus (Shepherd, 1979). During development, however, mitral cells initially extend dendrites into multiple glomeruli. These elaborations are gradually remodeled during postnatal stages until most mitral cells retain a single primary dendrite that innervates a single glomerulus (Malun and Brunjes, 1996). The olfactory bulb therefore provides a spatial map that can identify which of the numerous receptors have been activated within the sensory epithelium, such that the quality of an olfactory stimulus would be encoded by specific combinations of glomeruli activated by a given odorant.

The observation that each of ~1000 different subpopulations of sensory neurons project with precision to a small number of topographically fixed glomeruli poses an interesting and complex problem of axon targeting and synapse specificity. We have asked whether the specificity of connections between olfactory sensory axons and mitral cell dendrites is dependent upon odorant-evoked neural activity. Mice bearing a targeted mutation in the α subunit of the olfactory cyclic nucleotide–

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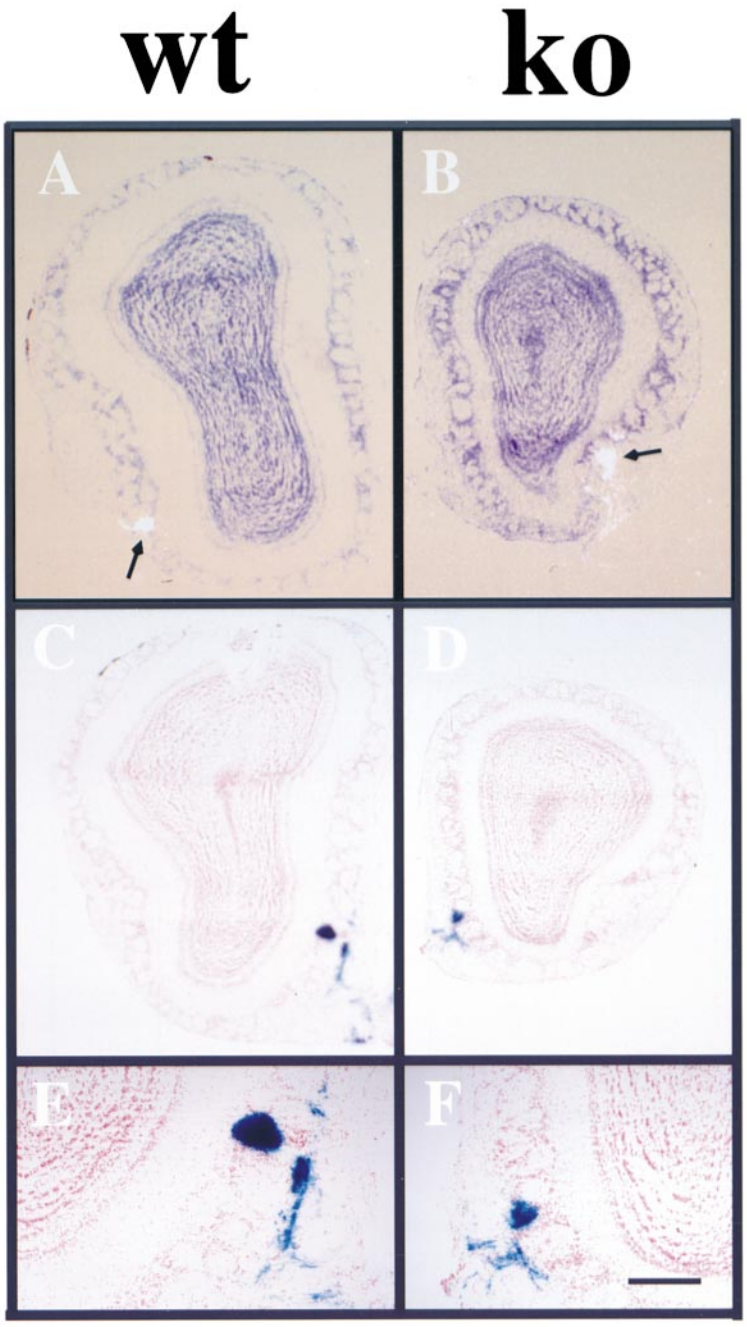

Figure 1. Convergence Occurs Normally in Mice Lacking Olfactory CNG Channel Function

(A and B) Coronal sections of adult olfactory bulbs hybridized with a ^{33}P-labeled probe for receptor M50. Tissue sections and hybridization signals were visualized by simultaneous bright-field/dark-field illumination. Hybridization over the M50 lateral glomerulus is observed in both wild-type (wt) and hemizygous CNG channel mutant (ko) mice (arrows). In these two examples, signals resembling comet tails can be observed within the olfactory nerve layer as M50-positive axons coalesce and enter the target glomeruli. The medial glomeruli receiving M50 receptor innervation occupy a different position along the anterior–posterior axis and therefore are not visible in either of these sections. Results similar to those shown here were obtained from a total of nine adult CNG channel knockout animals and eight wild-type animals.

(C and D) Coronal sections of adult olfactory bulbs from mice crossed onto the receptor P2-IRES-tau::lacZ "blue mouse" background, stained for β-galactosidase activity. Labeled axons can be seen as they converge to the medial glomerulus in both wild-type and hemizygous mutant animals.

(E and F) Higher power views of the labeled glomeruli shown in (C) and (D).

Scale bar, 1 mm for (A)–(D) and 200 μm for (E) and (F).

gated (CNG) ion channel, a critical component of the olfactory signaling cascade, fail to exhibit odorant-evoked responses to a wide range of odorant stimuli (Brunet et al., 1996). In this study, we demonstrate that the convergence of like axons in the olfactory bulb onto spatially invariant glomeruli is unaltered in these mutant mice. We further show that the pruning of mitral cell dendritic projections, although slowed during development, is ultimately indistinguishable in CNG mutant and wild-type animals. Thus, the olfactory CNG channel—and by inference correlated presynaptic neural activity—is not required for the establishment of the highly ordered synaptic connections that define an olfactory sensory map in the olfactory bulb.

Results

Olfactory Neuron Convergence Is Normal in Mice Lacking Functional Olfactory CNG Channels

We have shown previously that a null mutation in *Cnca*, the gene encoding the α subunit of the olfactory CNG channel, causes the elimination of odor-evoked excitatory responses in olfactory neurons (Brunet et al., 1996). In addition, most mutant mice die shortly after birth, probably due to an inability to nurse (Brunet et al., 1996). Nonetheless, it is possible to raise a subpopulation of mutants to adulthood, despite the persistent absence of odor responsivity. Based on the expression of a number of molecular markers, olfactory neurons

mature normally in the absence of functional olfactory CNG channels (Brunet et al., 1996). Histological analyses in adults indicate that the outermost olfactory nerve layer is present and glomerular structures are formed (Figure 1). However, the bulbs from adult hemizygous mutants are markedly smaller than those from control animals, with a decrease in the thickness of all cell layers (Figure 1). Similar results have been observed by others studying an independently derived null mutation in the *Cnca* gene (Baker et al., 1999). These observations are consistent with other studies that showed that a reduction of olfactory bulb size occurs following inhibition of odorant-evoked activity by naris occlusion (Brunjes, 1994).

The projection pattern of olfactory sensory neurons into the bulb of wild-type and mutant mice was assessed with two complementary experimental approaches. Previous studies have shown that RNA in situ hybridizations could detect odorant receptor mRNA in olfactory neuron axon termini (Ressler et al., 1994; Vassar et al., 1994). The M50 odorant receptor, for example, is expressed in neurons that converge to bilaterally symmetric glomeruli located on the medial and lateral surfaces of the olfactory bulb (Figure 1A; Ressler et al., 1994). In situ hybridization with the M50 receptor in CNG channel mutant adult mice detects a comparable pattern of glomerular convergence (Figure 1B). The locations of these sites of convergence are similar in all animals examined.

Previous studies have shown that neurons expressing a given odorant receptor can innervate one or two glomeruli at each site of convergence in the olfactory bulb (Mombaerts et al., 1996; Royal and Key, 1999). The proportion of sites that display one versus two receptor-specific glomeruli is variable, however, and in some instances the fraction of "double" glomeruli sites has been reported to be as high as 85% (Royal and Key, 1999). We find by in situ hybridization that M50 neurons converge to one or two closely situated M50 glomeruli on the medial and lateral aspects of the olfactory bulb in both wild-type and mutant animals. In eight wild-type animals examined, 64% $\pm$ 27% (mean $\pm$ standard deviation) of M50 convergence sites consist of a single glomerulus, and the remaining sites comprise two glomeruli (total of 29 sites scored). In nine hemizygous CNG channel mutants, 44% $\pm$ 31% of the M50 sites contain a single glomerulus, with the remaining sites containing a pair of glomeruli (total of 29 sites scored). Convergence to sites containing one or two spatially defined glomeruli is also observed with probes for the M71, A16, and P2 odorant receptors, although fewer animals were examined with these receptor genes (data not shown). These results indicate that, to a first approximation, the convergence of sensory axons to appropriate target glomeruli proceeds in the absence of odorant-evoked activity.

RNA in situ hybridization to specific receptor RNAs in axonal termini provides one indication of convergence, but does not allow us to follow the projection pattern of all neurons that express a specific odorant receptor. Smaller numbers of stray fibers that may have targeted to additional glomeruli, or perhaps failed to enter the glomerular layer altogether, would have escaped detection owing to the limited sensitivity of this method. We therefore employed a genetic approach in which the axons of cells expressing a specific odorant receptor are labeled with a tau::lacZ reporter (Mombaerts et al., 1996). Using gene targeting, a cassette that contains an internal ribosome entry site (IRES) directing the translation of the tau::lacZ fusion protein was inserted immediately downstream of the P2 odorant receptor stop codon (Mombaerts et al., 1996). Cells that express this modified *P2* allele now express the tau::lacZ marker (which can be visualized by histological staining for β-galactosidase activity) along with the P2 receptor. In these genetically altered strains of mice, neurons expressing the P2 receptor converge upon two topographically fixed sites in the mouse olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998). We crossed heterozygous female olfactory CNG channel mutant mice with homozygous male P2-IRES-tau::lacZ mice and examined the pattern of projections of P2 neurons in the offspring. P2 neurons that express lacZ converge to one or two glomeruli on the medial and lateral surfaces of the olfactory bulb in wild-type and CNG channel mutant mice (see Figures 1C and 1D). Moreover, no stray fibers are observed that project to regions outside of the P2 glomeruli.

Because of the dramatic differences in bulb size, it is not possible to determine whether specific receptor-expressing neurons in fact converge to the same glomeruli in wild-type and mutant adult mice. We observed that the reduction in olfactory bulb size in olfactory CNG channel mutants is not yet manifested in neonatal mice. It was therefore instructive to assess the relative positions of glomeruli in neonates expressing the *P2-IRES-tau::lacZ* allele. We analyzed olfactory tissue from pups obtained by crossing CNG channel mutant mice with P2-IRES-tau::lacZ mice. Snouts were bisected sagittally to reveal the olfactory turbinates and the medial surfaces of the olfactory bulbs. Staining of these whole-mount preparations for β-galactosidase activity reveals receptor P2–expressing cells in the olfactory epithelium, as well as their axonal processes, which can be observed as they project into the olfactory bulb and converge to the medial glomerulus (Figure 2). At this level of resolution, it appears that the blue axons converge to a region in the mutant bulb (Figure 2B) at a position comparable to that observed in the wild-type preparation (Figure 2A). Staining of tissue sections taken from other neonatal olfactory bulbs confirms that the receptor P2–expressing cells converge to one or two glomeruli on the lateral and medial aspects of the bulb (Figures 2C and 2D). In wild-type animals, 40% of the P2 convergence sites consist of a single glomerulus and 60% reveal two glomeruli ($n = 4$ animals, 15 sites scored), whereas in mutant mice, 67% of the P2 sites are single glomeruli and 33% consist of two glomeruli ($n = 5$ animals, 18 sites scored).

We also examined the relative positions of the M50 and P2 glomeruli in mutant and wild-type mice. Alternate serial sections from olfactory bulbs of P2-IRES-tau::lacZ adult mice were subjected to M50 in situ hybridizations or β-galactosidase staining. In bulbs from both wild-type and olfactory CNG channel mutant mice, the order along the anterior–posterior (A–P) axis is: lateral P2 glomerulus → lateral M50 glomerulus → medial P2 glomerulus → medial M50 glomerulus. In the hemizygous CNG channel mutant, we observed $\sim$200–300 μm spacing between the lateral P2 and lateral M50 glomeruli (vs. $\sim$280–340 μm in the wild type), $\sim$60–80 μm spacing between the lateral M50 and medial P2 glomeruli (vs.

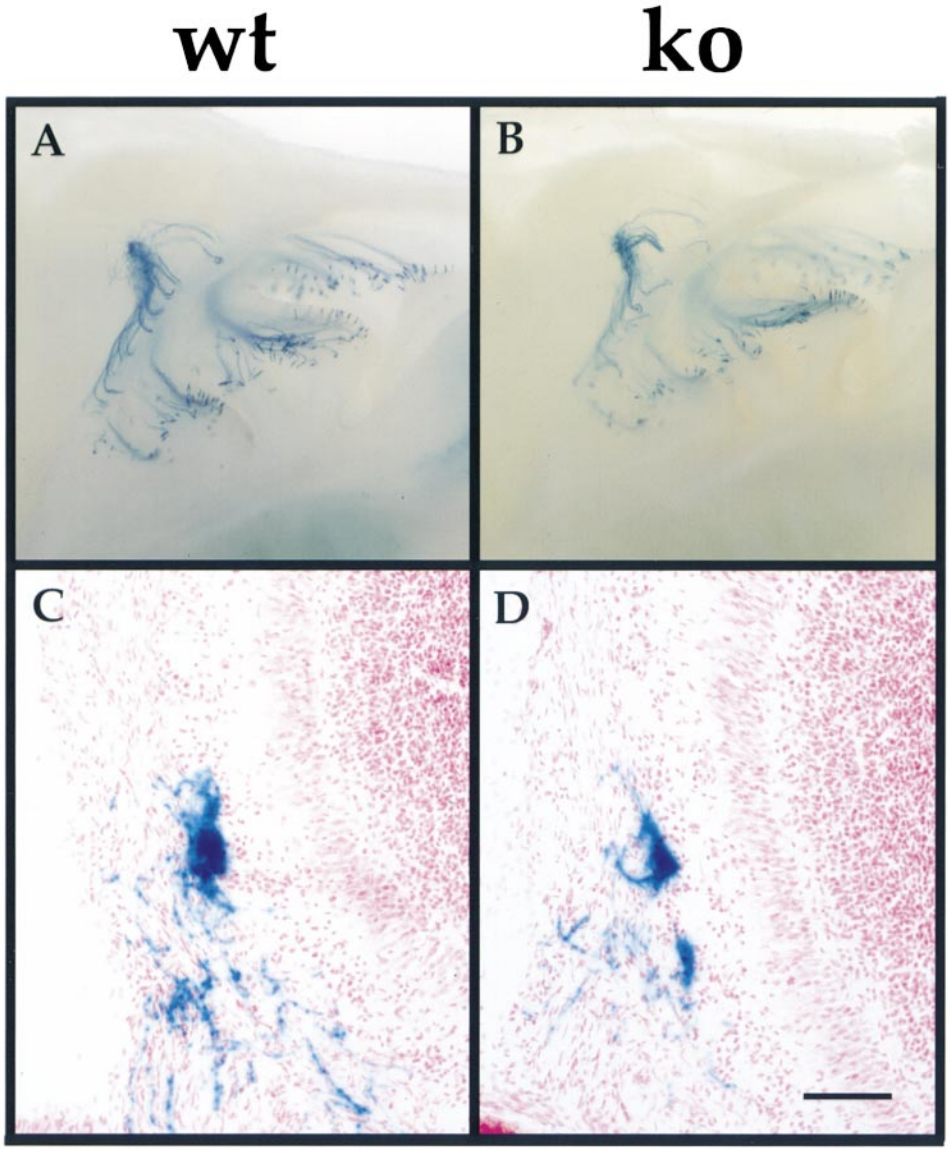

Figure 2. Analysis of Olfactory Bulb Convergence in Neonatal Mice

Snouts from 1-day-old wild-type (wt) and hemizygous mutants (ko) crossed onto the P2-IRES-tau::lacZ background were bisected sagitally and stained for β-galactosidase activity (A and B). In each case, blue axons can be seen as they stream from right to left from the lateral turbinates toward their medial glomerular target. The positions of these targets are indistinguishable between wild-type and mutant bulbs at this level of analysis. Staining for β-galactosidase activity in olfactory bulb tissue sections from 1-day-old mice similarly shows the convergence of labeled axons to single glomerular targets in both wild-type and mutant backgrounds (C and D). These results are representative of experiments carried out on a total of eight neonatal knockout animals (three animals by whole-mount staining and five animals by staining in tissue section) and a comparable number of control animals. Scale bar, 50 μm for (C) and (D).

∼80–100 μm in the wild type), and ∼200–280 μm spacing between the medial P2 and medial M50 glomeruli (vs. ∼260 μm in the wild type). Thus, taking into consideration the smaller overall sizes of olfactory bulbs in the mutant background, the relative spacing of these glomeruli appears to be similar in mutant and wild-type mice.

Convergence of Olfactory Neuron Axons in the Olfactory Bulb Is Not Dependent on Embryonic Odorant Receptor–Mediated Activity

The results presented thus far suggest that odorant-evoked activity is not required for the precise convergence of olfactory neurons in the olfactory bulb. We have previously shown that neonatal mice deficient in olfactory CNG channels fail to exhibit odorant-evoked responses in the olfactory epithelium (Brunet et al., 1996). Since convergence of like axons in the olfactory bulb is apparent as early as embryonic day 16.5 (E16.5; Mombaerts et al., 1996; Wang et al., 1998; Royal and Key, 1999), it is important to demonstrate the absence of odorant-evoked responses during these early developmental stages. It is possible, for example, that another CNG α subunit gene is expressed embryonically and then downregulated at birth. We therefore performed

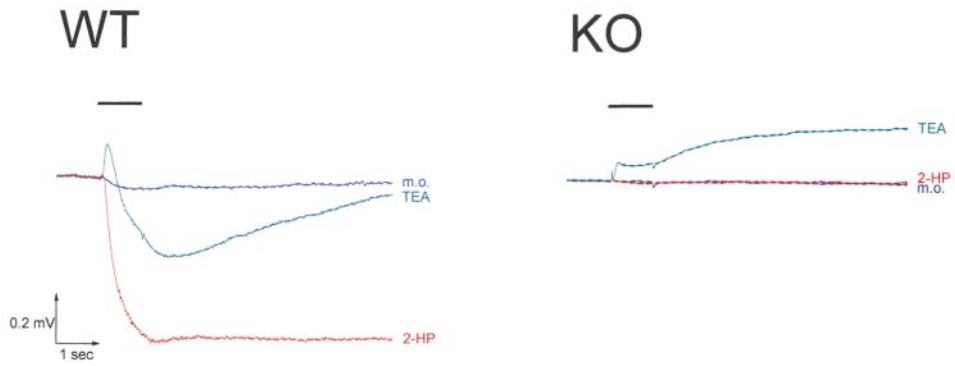

Figure 3. Absence of Odorant Receptor-Mediated Activity in Embryonic Mice Hemizygous for the Olfactory CNG Channel Mutation

EOG responses were measured from olfactory epithelia from wild-type (WT) and hemizygous mutant (KO) E17.5 embryonic mice. Each panel is an overlay of successive traces recorded from an individual embryo and shows responses to mineral oil (m.o.), 2-hexylpyridine (2-HP), and triethylamine (TEA). Each odorant stimulus was applied for 1 s (black bar over each set of traces). Note that the slow monophasic responses to mineral oil and 2-HP normally observed in the control epithelium are undetectable in the mutant preparation. Of the biphasic response normally seen with TEA, only the rapid potential of opposite polarity remains in the mutant; this response is of nonneuronal origin and therefore does not reflect a receptor potential (Okano and Takagi, 1974; Brunet et al., 1996). For these experiments, a total of three hemizygous mutant embryos (from two separate litters) were identified by genotyping; none of these mutants exhibited a response to any odorant tested, except for the rapid TEA response with opposite polarity.

electroolfactogram (EOG) recordings, which measure the locally summated activity of neurons in the olfactory epithelium (Ottoson, 1956), from wild-type and mutant mice at 17.5 days of embryonic development (E17.5). As shown in Figure 3, 2-hexylpyridine, triethylamine (TEA), and mineral oil elicit slow responses in wild-type embryonic epithelium. The response to TEA is biphasic, with a rapid potential of opposite polarity preceding the slow potential. This initial, rapid response is thought to arise from secretory cell activity, whereas the second phase is neuronal in origin (Okano and Takagi, 1974; Brunet et al., 1996).

As demonstrated previously using neonatal preparations (Brunet et al., 1996), embryos hemizygous for the CNG channel mutation show no detectable odorant-evoked responses, with the exception of the initially rapid and then prolonged TEA response of a polarity opposite to that of the other odorant-evoked responses (Figure 3). Since this TEA response is nonneuronal in origin, it also serves as a positive control for the EOG recording itself (Brunet et al., 1996). Testing with other odorants (citralva, isomenthone, isovaleric acid, lilial, and pyrazine) similarly reveals EOG responses in wild-type, but not in mutant, embryos (data not shown). Although we did not observe any measurable EOG responses in mutant embryos (aside from the nonneuronal TEA response of opposite polarity), we estimate that potentials as small as $\sim$1%–3% of wild-type responses, if present, should have been detectable. This estimate is based on the noise of the EOG recordings ($\sim$0.01–0.02 mV) divided by the peak amplitudes observed in recordings from wild-type preparations (up to $\sim$0.7–0.8 mV) and sets an upper limit for the smallest response that can be reliably detected (see Brunet et al., 1996). Together our observations demonstrate that receptor-mediated signaling does not occur at embryonic stages in mice lacking the olfactory CNG channel α subunit gene and argue against the possibility that such signaling could play a role in the genesis of olfactory neuron convergence in the olfactory bulb.

Postnatal Remodeling of Olfactory Bulb Mitral Cell Dendrites

The glomerular neuropil is formed from the interaction of olfactory sensory neuron axons with mitral, tufted, and periglomerular neuronal processes (Shepherd, 1979). Mitral cells initially extend dendrites into multiple glomeruli. During postnatal development, these dendrites are gradually retracted and remodeled until most mitral cells possess a single dendrite that innervates a single glomerulus (Malun and Brunjes, 1996). Since the olfactory sensory map forms prior to the remodeling of mitral cell dendritic arbors, correlated neural activity within individual glomeruli could play a role in the dendritic pruning process. We therefore examined this remodeling process in wild-type and olfactory CNG channel mutants to determine whether odorant-evoked activity in olfactory sensory neurons is required for the refinement of mitral cell dendrites.

Mitral cells from neonatal wild-type pups were retrogradely labeled by application of the lipophilic tracer dye, dil, to the lateral olfactory tract. After allowing time for the dye to diffuse back to the mitral and tufted cell bodies and dendrites, bulbs were sectioned and cells were visualized by confocal fluorescence microscopy. While both mitral and tufted cells could be readily identified by this method, we focused our studies primarily on mitral cells. In accord with previous work (Hinds and Ruffett, 1973; Santacana et al., 1992; Malun and Brunjes, 1996), we find that significant remodeling of mitral cell dendritic arbors occurs in the mouse during early postnatal development. At birth (P0), nearly all mitral cells possess multiple dendrites that extend radially from the cell body into the external plexiform layer. These dendrites lack any tufted specializations at their termini and are uniform in thickness and in length (Figure 4A). At 3 days of age (P3), mitral cell dendrites have increased in length and appear to contact several glomeruli. In many cells, a single primary dendrite could be identified that was thicker than the surrounding dendrites (Figure 4B). This thicker dendrite was also found to possess a rudimentary tuft at its terminal projection, while surrounding

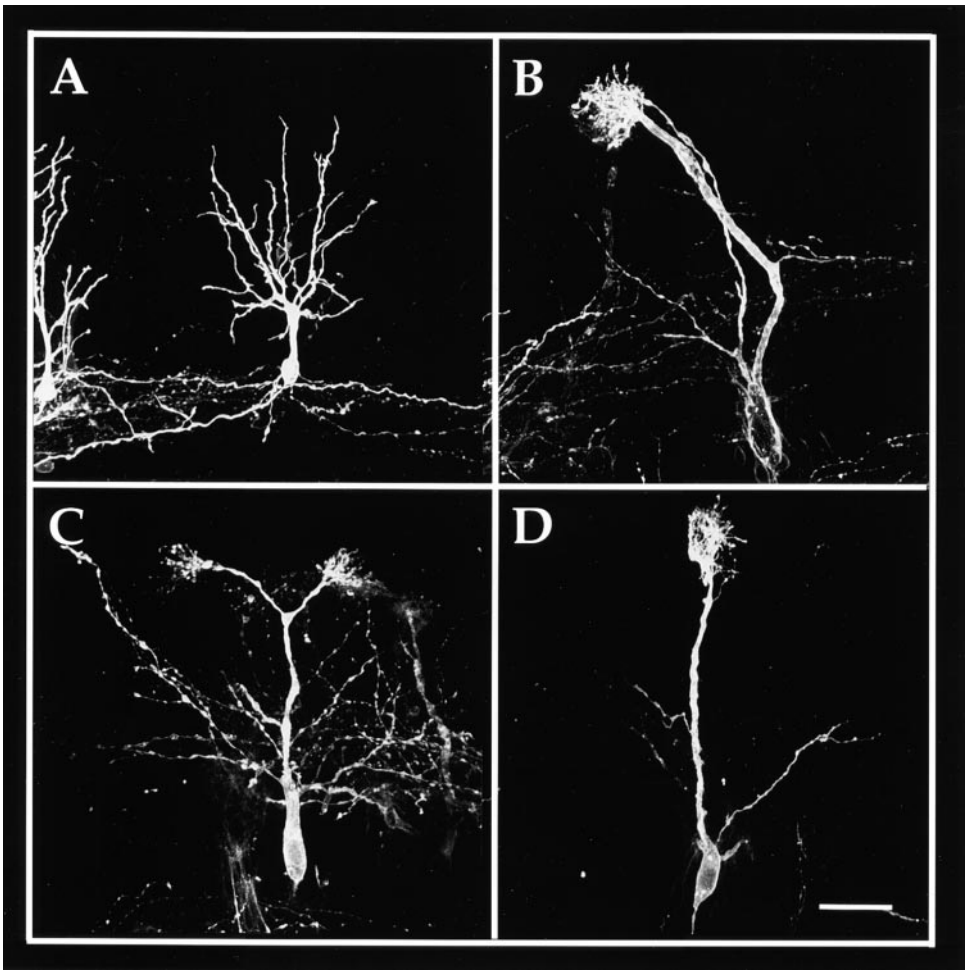

Figure 4. Refinement of Mitral Cell Dendritic Arbors during Development

The morphology of mitral cells from neonatal (P0–P6) wild-type mouse olfactory bulbs was assessed by dil-labeling and fluorescence confocal microscopy. Images were prepared by brightest point projection of serial confocal optical sections.

(A) Mitral cells with immature morphologies possess numerous uniform and widespread dendrites.

(B) As refinement of arbors proceeds, dendrites increase in length. One dendrite often appears thicker than the others and possesses a terminal tuft.

(C) A relatively striking observation is the number of mitral cells with developing primary dendrites in two or more distinct glomeruli. This is probably a transition phenotype, as the proportion of mitral cells with this appearance drops during development.

(D) Mature mitral cells possess a single, thick primary dendrite with a terminal tuft that ramifies within a single glomerulus.

Secondary dendrites can be seen as they radiate within the external plexiform layer (horizontally in these micrographs). Scale bar, 50 μm.

dendrites often only had simple, relatively sparse endings (Figure 4B). The apparent selection of one process to become the primary dendrite is accompanied by the gradual remodeling of the surrounding apical dendrites. During this period, a wide variety of transition phenotypes could be observed. In several instances, mitral cells were observed that have two primary dendrites in contact with two glomeruli (Figure 4C). By P6, most mitral cells possess a single, primary dendrite with a well-developed tuft that innervates a single glomerulus (Figure 4D). Some mitral cells, however, still maintain contact with two or more glomeruli. In the adult, the vast majority of mitral cells (>85%) exhibits a single primary dendrite (see Table 1).

We next examined the pruning of mitral cell dendrites in olfactory CNG mutant mice to determine whether odorant receptor-evoked activity is required for this refinement process. As in wild-type mice, at P0 nearly all mitral cell dendrites in CNG channel mutants appear immature (Figure 5A). In P3 mutant pups, mitral cells have clearly begun to refine their dendritic arbors (Figures 5B and 5C). Retraction of dendrites and selection of a primary dendrite appear to parallel that of their heterozygous or wild-type littermates (Figure 5B vs. Figure 4B). Mitral cells with single primary dendrites are also clearly present at this age (Figure 5D). In slightly older P4 and P6 mutant pups, however, the proportion of mature mitral cells with single primary dendrites appears to be less than that of the controls. To determine whether these impressions were correct, we quantified the rate at which mitral cell dendritic development proceeds in normal and mutant mice (Table 1). Individual

Table 1. Distribution of Mitral Cells Showing Multiple, Double, or Single Glomerular Innervation in Wild-Type and Olfactory CNG Channel Mutant Mice[a]

Stage	Multiple	Double	Single	Total Number of Cells Scored
Wild type/heterozygous				
P0	94.3%	4.9%	0.8%	123
P3	67.3%	19.6%	13.1%	93
P4	23.0%	25.0%	52.0%	100
P6	5.0%	23.8%	71.3%	80
Adult	0%	12.1%	87.9%	91
Hemizygous mutant				
P0	96.4%	3.6%	0%	56
P3	66.0%	20.8%	13.2%	144
P4	48.9%	27.7%	23.4%	47
P6	19.3%	38.5%	42.2%	109
Adult	0%	14.9%	85.1%	47

[a] Mitral cell dendrites were visualized and scored following retrograde labeling with dil. Data tabulated are from experiments conducted on the 129/Sv inbred background.

mitral cells were categorized into three classes based upon dendritic morphology: (1) cells exhibiting multiple (greater than 2) dendrites extending to the glomerular layer (e.g., Figures 4A and 5A); (2) cells with two main dendrites (Figures 4C and 5C; because of the wide variety of phenotypes observed during remodeling, this strictly defined category identifies mitral cells that appear to be near the end of the maturation process); and (3) cells possessing a single primary dendrite whose terminals ramified within a single glomerulus (Figures 4D and 5D).

The data tabulated in Table 1 confirm our qualitative impressions regarding the development of mitral cell dendrites in CNG channel knockout mice. In the hemizygous mutant background, the rate of refinement from P0 to P3 is indistinguishable from wild-type or heterozygous controls but appears to slow between P4 and P6. Nonetheless, the proportion of mature mitral cells (as determined by the percentage of mitral cells with a single dendrite) at P4 and P6 is still greater than at P3 in the mutant background. To determine whether dendritic development simply slows after P4 or, alternatively, has reached a plateau, mitral cell morphologies were scored in adult mice. No difference in the proportion of mitral cells exhibiting the three defined dendritic morphologies could be seen between hemizygous mutant and heterozygous or wild-type adults (Figures 6A–6C; Table 1). An identical series of experiments was carried out with mutant mice bred in a different isogenic background and produced similar results (data not shown; see Experimental Procedures). Thus, the maturation of mitral cell dendrites appears to be largely unaffected by the absence of odorant-evoked activity. The reduced rate of pruning observed from P4 to P6 may be due to the absence of odorant-evoked activity but is more likely the result of secondary developmental defects. Mutant pups suckle poorly and are easily recognizable among littermates due to their smaller size (Brunet et al., 1996). By P6, if they survive, they often weigh three to four times less than their heterozygous or wild-type littermates (D. M. L., L. B., and J. N., unpublished data). The apparent slowing of dendritic refinement in P4–P6 hemizygous mutants may therefore be a consequence of the slower overall growth rate of the entire animal. Whatever the cause, greater than or equal to 85% of mitral cells exhibit a single radial dendrite in both wild-type and mutant adults mice, strongly suggesting that odorant-evoked activity is not required to achieve the mature patterning of mitral cell dendritic arbors.

Discussion

Convergence of Olfactory Neurons Occurs in the Absence of Odorant-Evoked Activity

In the olfactory system, sensory neurons expressing a given odorant receptor project with precision to two spatially invariant glomeruli within the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998). This observation raises the question as to how topographically disparate cells in the periphery converge to establish a sensory map in the bulb. In one model, spatially restricted guidance cues in the bulb are the sole determinants specifying an invariant pattern of connections of olfactory sensory neurons (see Tessier-Lavigne and Goodman, 1996). Other models invoke activity-dependent processes that operate in concert with target-derived guidance cues to achieve the precision of connections between the olfactory neurons and the bulb. In one such model, spontaneous or evoked activity in olfactory sensory neurons might play a permissive role in the guidance process, such that the firing of sensory neurons would be required to allow a genetically encoded targeting process to unfold. Activity might also play a permissive role in the strengthening or stabilization of synapses once formed within appropriate glomeruli. In an alternative model, the correlated firing of olfactory neurons could play an instructive role in the establishment of a sensory map. Olfactory neurons expressing the same odorant receptor are dispersed in the sensory epithelium yet converge upon fixed targets in the olfactory bulb (Ressler et al., 1993, 1994; Vassar et al., 1993, 1994; Mombaerts et al., 1996; Wang et al., 1998). The excitation of like axons, producing a correlated pattern of neural activity, is therefore likely to require odorant receptor-evoked excitation of sensory neurons. In this manner, odorant receptor-evoked activity may play an instructive

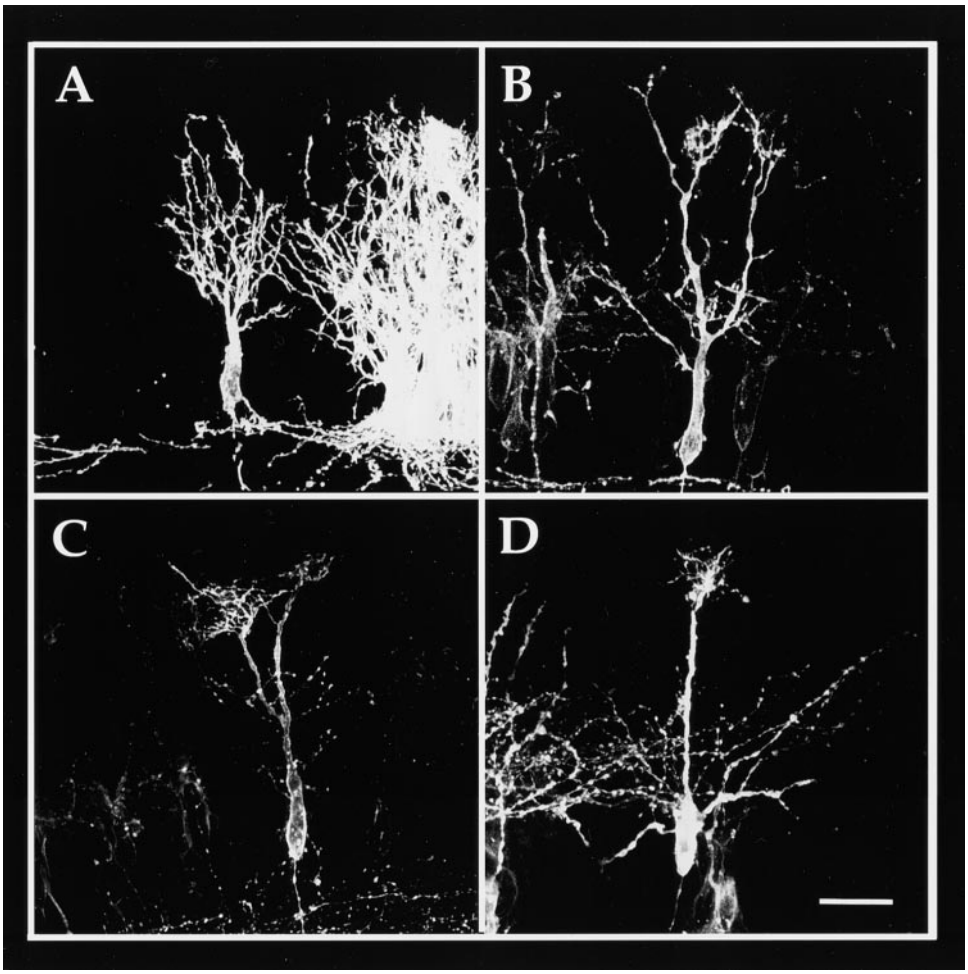

Figure 5. Refinement of Mitral Cell Dendritic Arbors Occurs in the Absence of Evoked Activity

Confocal micrographs of dil-labeled mitral cells from olfactory bulbs from neonatal (P0–P6) olfactory CNG channel hemizygous mutants are shown in this figure. The refinement of dendritic arbors appears to parallel that of wild-type mice (see the text).

(A) Immature mitral cells possess multiple dendrites (an isolated cell in the center of this micrograph can be observed near a densely labeled group of cells to the right). Selection of a primary dendrite with a thickened appearance generates several transition phenotypes, including innervation of more than one glomerulus (B and C). (D) Mature mitral cell with a single, primary dendrite. Scale bar, 50 μm.

role in the generation and refinement of a topographic map.

In this study, we address the potential role of correlated neuronal activity in the establishment of the olfactory sensory map. Mice lacking the olfactory CNG channel fail to exhibit odorant-evoked electrophysiologic responses to a wide range of odor stimuli. We nonetheless find that the convergence to appropriate target glomeruli by cells expressing either the M50 or P2 odorant receptors is unaffected in CNG channel mutant mice. The relative spacing and order of the M50 and P2 glomeruli are maintained, suggesting that other olfactory neuron populations are probably also unaffected in the mutant background. These data argue strongly that olfactory experience is not required for the establishment of precise patterns of afferent innervation in the olfactory bulb. Since it is difficult to envisage a mechanism to correlate the activity of randomly distributed neurons expressing the same receptor without odor-evoked stimulation of receptors, our data further imply that correlated neural activity is not required for the generation of the olfactory sensory map. Our results are consistent with previous studies that showed that mice deficient in the olfactory-specific G protein, G_{olf}, exhibit normal patterns of olfactory neuron convergence in the olfactory bulb (Belluscio et al., 1998). However, residual odorant-evoked activity in neonatal G_{olf}-deficient mice precluded a definitive conclusion with regard to the role of neural activity in the patterning of synaptic connections in the olfactory bulb.

The conclusion that olfactory experience and correlated neuronal activity are not required for synaptic patterning in the olfactory bulb should be tempered by the following considerations. First, despite the maintenance of the relative spacing between P2 and M50 glomeruli in mutant and wild-type mice, we cannot discern with absolute certainty whether the points of convergence are indeed identical in both genetic backgrounds. Second, we frequently observe multiple glomeruli at the sites of convergence in both wild-type and mutant bulbs. Analysis of the projections of M50 neurons reveals a greater frequency of "double" glomeruli than is ob-

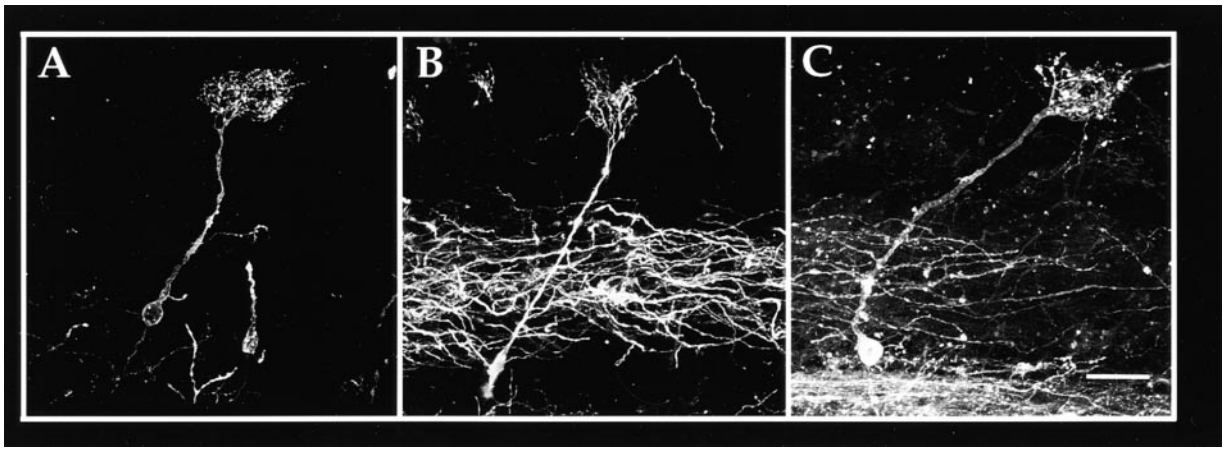

Figure 6. Dendritic Arbors in Adult CNG Channel Mutant Olfactory Bulbs

Mitral cell dendritic projections from 3-week-old mice hemizygous for the CNG channel mutation were visualized by retrograde DiI labelings. (A) A cell with a single primary dendrite that ramifies in two adjacent glomeruli can occasionally be observed. This cell is probably a tufted rather than a mitral cell. However, tufted cell dendritic morphologies develop in a similar fashion to mitral cells.
(B and C) Individual cells with single primary dendrites, each innervating a single glomerulus.
Scale bar, 50 μm.

served in wild-type animals, although this difference is not statistically significant. Indeed, we also find that P2 neurons converge to comparable proportions of single and double glomerular sites in mutant and wild-type bulbs. Additional data will be required to evaluate whether the CNG channel mutation does indeed influence the ultimate number of glomeruli at each convergence site. Whatever the outcome, the relative positions of glomeruli are maintained in mutant mice, and the differences in glomerular number are subtle. Moreover, we do not know whether the appearance of secondary glomeruli is physiologically meaningful or whether it results from errors in the targeting process that may reflect dramatic differences in basal neuronal physiology between mutant and wild-type mice. Third, we cannot rule out the possibility that activity-dependent synaptic competition underlies a refinement process during convergence. In the mammalian visual system, diffuse and overlapping inputs are observed when activity from both eyes is eliminated, but when activity from one eye remains, columns from the active eye occupy a greater area than those from the inactive eye (Hubel et al., 1977; LeVay et al., 1980; Stryker and Harris, 1986). In the mutants we have generated, evoked activity is eliminated in all olfactory neurons. Thus, the projection of olfactory neurons lacking evoked activity could be affected by the presence of active cells, as might normally occur when cells expressing one odorant receptor are activated by an odor while cells expressing other receptors are quiescent. Finally, it should be emphasized that our data do not address the possibility that spontaneous, noncorrelated activity or neurotransmitter release may be required in a permissive fashion for the establishment or maintenance of the spatial map.

Our observation that correlated neural activity does not play a significant role in the formation of the olfactory sensory map underscores the importance of the molecules that might direct this developmental process. Genetic experiments suggest that the odorant receptor, as one of a complement of other guidance molecules, plays a role in the establishment of patterns of olfactory neuron convergence in the olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998). Deletion or nonsense mutations in a given receptor gene cause axons of cells expressing these mutant alleles to wander broadly in the bulb without converging on a specific glomerulus (Wang et al., 1998). Receptor substitutions that replace the coding region of one receptor gene with that of another uniformly alter the pattern of convergence (Mombaerts et al., 1996; Wang et al., 1998). Together these results indicate that odorant receptors are required for olfactory neurons to extend their axons to the correct glomerular target, perhaps by recognizing specific guidance cues (see also Lin and Ngai, 1999). Our findings with the CNG channel knockout mouse argue that, to the extent that odorant receptors are involved in this process, they must operate via a signaling pathway independent of CNG channel function, and therefore independent of the sensory transduction pathway employed by these cells to elicit stimulus-evoked changes in membrane potential.

The Role of Neuronal Activity in Mitral Cell Dendritic Development

During development, mitral cells—the major class of projection neurons in the bulb—initially extend dendrites into multiple glomeruli. In mammals, extensive remodeling occurs postnatally, such that most mitral cells retain a single primary dendrite that arborizes extensively within a single glomerulus (Santacana et al., 1992; Malun and Brunjes, 1996; but see Pomeroy et al., 1990). These observations pose a second problem in the genesis of synaptic specificity in the olfactory system: how is the dendritic arbor refined to assure that a given mitral cell will synapse within only one glomerulus? In one model, the ultimate target of a given mitral cell dendrite is predetermined by genetically encoded guidance or survival cues that specify the pruning process. An alternative

model postulates that one of multiple dendritic arbors may be stochastically chosen for synapse formation or stabilization, and this positive choice sends a negative signal to other dendrites, resulting in their retraction. We have demonstrated that both wild-type and olfactory CNG mutant mice initially elaborate multiple dendrites, and these dendrites are ultimately pruned postnatally, such that in the adult the vast majority of mitral cells extend a single dendrite that arborizes within only one glomerulus. Whatever the mechanism underlying this refinement, our observations indicate that odorant-evoked activity, and by inference correlated presynaptic neuronal activity, is not required for the pruning of postsynaptic mitral cell radial dendrites.

The relative insensitivity of dendritic remodeling to neuronal activity has also been noted in the visual system. In the retina, ganglion cell dendrites are initially multistratified but eventually segregate into two distinct laminae reflecting the functional formation of ON and OFF pathways (Bodnarenko and Chalupa, 1993). Despite the apparent elaboration and refinement of ganglion cell dendrites (a pattern similar to that observed in mitral cells), application of TTX (Wong et al., 1991; Campbell et al., 1997), monocular occlusion, or dark-rearing (Leventhal and Hirsch, 1983; Lau et al., 1990) do not appear to perturb this process. These observations suggest that the control of postsynaptic dendritic branching in the visual system is independent of correlated or evoked neuronal activity. However, the perfusion of pharmacologic antagonists of the NMDA receptor alters the normal pattern of dendritic maturation in the retina (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995; Bisti et al., 1998) as well as in the LGN and tectum (Rocha and Sur, 1995; Rajan and Cline, 1998), suggesting that either spontaneous activity or spontaneous neurotransmitter release is required for dendritic remodeling (see also McAllister et al., 1996).

Is Activity-Dependent Synaptic Refinement a General Feature of CNS Development?

The precision of synaptic connections in the vertebrate central nervous system is thought to depend critically on genetically encoded guidance cues, as well as correlated neural activity (Shatz, 1990; Goodman and Shatz, 1993; Katz and Shatz, 1996). In the olfactory system, however, correlated presynaptic activity does not appear to be required either for the targeting of olfactory neuron projections in the bulb or for the refinement of mitral cell dendrites. These data strongly suggest that the primary determinants underlying the genesis of this sensory map are genetically encoded spatial cues. Molecular cues are also likely to play an important, if not predominant, instructive role in the generation of visual and somatosensory representations in the brain. In the mammalian visual system, eye-specific retinotopic maps in the thalamus and superior colliculus are thought to result from a graded set of guidance cues, the ephrins, elaborated by their targets and recognized by ephrin receptors on ingrowing axons (Feldheim et al., 1998; Frisén et al., 1998). The role of neuronal activity in the generation and refinement of the retinotopic map during development has been difficult to establish experimentally (e.g., see Stuermer et al., 1990; Hubener and Bonhoeffer, 1999). In the somatosensory system, the formation of a coarse somatotopic map, represented in cortical whisker barrels, proceeds despite the blockade of neural activity. However, pharmacologic and genetic manipulations have suggested that finer scale projections, as well as plasticity, proceed through an activity-dependent mechanism(s) (Schlaggar et al., 1993; Li et al., 1994; Fox et al., 1996; Glazewski et al., 1996). The primary determinants driving the formation of sensory maps are therefore likely to rely heavily on genetically programmed interactions between guidance cues and their receptors on sensory axons. Once sensory maps are established, however, activity-dependent plasticity is likely to be required to allow the organism to respond to a changing sensory environment (Buonomano and Merzenich, 1998; Sur et al., 1999). Thus, the genesis of specific synaptic connections during development may indeed employ different mechanistic principles than those required for maintenance and plasticity later in life.

Experimental Procedures

Olfactory CNG Channel Mutant Mice

Mice harboring a targeted mutation in *Cnca*, the gene encoding the α subunit of the olfactory CNG ion channel (Brunet et al., 1996), were used in this study. We refer to this gene as *Cnca*, according to the designation assigned by the Mouse Genome Database Mouse Nomenclature Committee. As the *Cnca* gene is X linked, males carrying a copy of the mutated gene are hemizygous null mutants and were used to analyze the potential effects of the targeted mutation in the *Cnca* gene. Genotyping was performed as described previously (Brunet et al., 1996), either by genomic Southern blotting or by PCR for the *neo* and *sry* genes (indicating the presence of the targeted mutation and the Y chromosome, respectively).

In Situ Hybridization

Male mice hemizygous for the targeted olfactory CNG channel mutation were derived by crossing female heterozygous mutants with wild-type males (Brunet et al., 1996). Coronal sections were prepared from olfactory bulbs of hemizygous mutant and wild-type adult mice derived on either the 129/Sv or C57BL/6 background. Localization of odorant receptor RNAs was performed on 20 μm–thick fresh frozen sections using a ^{33}P-labeled antisense RNA probe for mouse odorant receptor M50 (Ressler et al., 1993), essentially as described (Wilkinson et al., 1987).

β-Galactosidase Staining

Male mice homozygous for the receptor *P2-IRES-tau::lacZ* allele (Mombaerts et al., 1996) were crossed with female mice heterozygous for the olfactory CNG channel mutation. Roughly one-half of the resulting male progeny were therefore heterozygous for the receptor *P2-IRES-tau/lacZ* allele and hemizygous for the olfactory CNG channel mutation. Histochemical staining for β-galactosidase activity from the tau::lacZ reporter was performed on adults and 1-day-old pups as described (Mombaerts et al., 1996).

EOG Recordings

Olfactory CNG channel heterozygous mutant females were mated with wild-type males, and gestation was allowed to proceed to 17.5 days. Embryos were harvested and decapitated, and heads were bisected sagitally to reveal the medial surfaces of the olfactory turbinates. Bisected heads were kept in oxygenated Ringers solution to preserve the sensory epithelium prior to recordings. EOG recordings were performed essentially as described previously (Wang et al., 1993; Brunet et al., 1996). Olfactory epithelia were

exposed either to a clean stream of humidified oxygen (100% oxygen before humidification) or to an odorized vapor stream that was made by passing humidified oxygen through a horizontal glass cylinder half-filled with odorant solution. The odorants used were mineral oil, 2-hexylpyridine (10^{-3} dilution in mineral oil), isomenthone (10^{-3} dilution in mineral oil), citralva (10^{-3} dilution in mineral oil), lilial (10^{-2} dilution in mineral oil), triethylamine (10^{-3} dilution in mineral oil), isovaleric acid (0.02 M in water), and pyrazine (0.01 M in mineral oil). In these embyronic preparations, the polarity of the odorant-induced EOG potentials was consistently reversed as compared to similar recordings we have documented previously in neonatal mice, where the odorant-induced neuronal potential is negative (Brunet et al., 1996). While we do not have a quantitative analysis of this polarity reversal, it may be explained qualitatively as being due to a layer of residual Ringers solution that remains on the heads as they sit on the recording dish. Because of surface tension, this layer is substantially thicker relative to the embryonic head as compared to the layer typically covering a neonatal head, and it tends to short-circuit the reference electrode with respect to the surface of the turbinates. Thus, the field potential seen by the reference electrode would be dominated by the negative potential over the turbinates, to the extent that it is more negative than the field potential seen by the recording electrode. The waveforms shown in Figure 3 were inverted to be consistent with our previously published studies (Brunet et al., 1996).

Mitral Cell Labeling

Heterozygous 129/Sv female mice bearing a targeted disruption of the gene encoding the α subunit of the cyclic nucleotide gated channel (Brunet et al., 1996) were crossed to wild-type 129/Sv males. Resulting progeny were screened by PCR for the presence of the neomycin resistance gene and the Y chromosome–specific *Sry* gene (Gubbay et al., 1992; see Brunet et al., 1996). Hemizygous mutant males, heterozygous females, and wild-type pups from various postnatal stages (P0 = first 24 hr after birth) were sacrificed by decapitation. Brains with attached olfactory bulbs were removed from the skull and fixed for a minimum of 24 hr in a 0.1 M phosphate-buffered solution of 4% paraformaldehyde. A small incision was made in the presumptive lateral olfactory tract, and a small crystal of DiI $C_{18}(3)$ (Molecular Probes, Eugene, OR) was applied. Brains were replaced in fixative, wrapped in aluminum foil, and incubated at room temperature for 4–6 days (P0–P6 pups). Adult brains were incubated at 37°C for 2–3 weeks. Brains were then removed from fixative and embedded in 2% low-melt agarose, and 100 μm–thick vibratome sections were collected (Oxford Model G). Sections were cleared in 70% glycerol and imaged with a Nikon PCM-2000 fluorescence confocal microscope. Z sections through selected mitral cells were imported into the NIH Image software package and projected in the x axis to generate brightest point projection patterns. An identical series of experiments was performed with mutant pups bred on the C57BL/6 background and compared to heterozygous or wild-type C57BL/6 mice. Results similar to those obtained with 129/Sv mice were observed, with the exception that the rate of mitral cell dendritic maturation is somewhat faster in the C57BL/6 strain.

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